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Interlaboratory Root Elongation Testing of Toxic Substances on Selected Plant Species

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INTERLABORATORY ROOT ELONGATION TESTING OF TOXIC SUBSTANCES ON SELECTED PLANT SPECIES

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INTRODUCTION

The Environmental Protection Agency's Office of Pesticides and Toxic Substances (OPTS) recommends test standards for determining the toxicity of new chemicals. Therefore, the OPTS must know the precision and expected variability of test results. Short term (115 hour) root elongation tests were performed to yield data on the acute phytotoxicity of chemical substances. This test is recommended as a bioassay to test environmental effects under the Toxic Substances Control Act (TSCA).

Four contract laboratories and three EPA laboratories participated in the interlaboratory testing (see Appendix A). None had previous experience in performing the test, and there was a wide variation in the testing experience of the different laboratories. The two commercial laboratories had considerable chemical testing experience but little or no familiarity with plant bioassays. The two University laboratories were strong in plant sciences but were less experienced with chemical testing. Each of the EPA laboratories had experience in performing tests using plant species and testing chemicals.

The ten toxic chemicals tested included two inorganic metals, an inorganic non-metal, an organometallic compound, two herbicides, a fungicide, an insecticide, an osmoticum, and an allelopathic substance. Most of the chemicals were readily soluble in water and had low acute mammalian toxicity. Only silver nitrate and endosulfan had LD50's (rat) below 50 mg/kg. Some of the chemicals, specifically endosulfan and silver nitrate, were included for comparison with the results of algal assays, fish acute toxicity tests and the Daphnia acute toxicity test.

Representatives of five different plant families (both monocot and dicot) were tested. These species are important economically as food and forage and important ecologically in terms of the family size, distribution, and abundance. Herbicide bioassays (Behrens, 1970; Horowitz, 1976; Santelmann, 1972), heavy metal screening tests (Konzak, 1976; Siegel, 1977; Whalley et al., 1974; Wilkins, 1957), salinity and mineral stress tests (Durrant et al., 1974; Neiman and Poulsen, 1971), and allelopathic studies (Asplund, 1969; Garb, 1961; Muller, 1965) have shown these plants to be sensitive to many toxic compounds. Each species was selected to represent either a root, leaf, seed, fruit or forage crop directly or indirectly consumed in the human food chain. The plants selected germinate quickly and easily, root growth is rapid and relatively uniform, and the cultivars are readily available.

This report presents a summary of the test data from seven participating laboratories, each testing 10 chemicals, and presents a method of calculating an EC50 from the dose-response data.

MATERIALS AND METHODS

Test Chemicals. Test compounds were selected to represent different classes of chemicals and to include pesticides and other substances with unknown phytotoxic properties. Root elongation tests were conducted with three inorganic compounds, silver nitrate ($AgNO_3$), sodium fluoride (NaF), and cadmium chloride ($CdCl_2$) (all from J. T. Baker Chemical Company, reagent grade); an organometallic compound, methane arsonic acid (MAA, Vineland Chemical Company, Vineland, New Jersey); and six organic compounds including endosulfan (Velsicol Chemical Corporation, Chicago, Illinois), 2,4-dichlorophenoxyacetic acid (2, 4-D, Dow Chemical Company, Midland, Michigan), polyethylene glycol 20,000 linear (Carbowax®, Union Carbide Corporation, New York, New York), pentachloronitrobenzene (PCNB), cineole (eucalyptus oil, Aldrich Chemical Company, San Leandro, California) and monuron (Hopkins Agricultural Chemical Company, Madison, Wisconsin). The organometallic and organic compounds were of technical grade, with the exception of cineole. Each chemical supplied to the laboratories was from a common chemical lot.

Stock solutions of $AgNO_3$, NaF, $CdCl_2$, Carbowax, and MAA were prepared in distilled water. Stock solutions were diluted with distilled water to obtain nominal concentrations of the test substance. A stock solution of 2,4-D was prepared by dissolving 2.0 g of 2,4-D and 0.36 g of NaOH in 1 liter of distilled water. Saturated solutions of endosulfan, PCNB, monuron, and cineole were prepared for use as test solutions in distilled water at 25°C. If saturated solutions significantly inhibited root length, subsequent dilutions were prepared to obtain nominal test concentrations. All stock solutions were prepared in glass or brown polyethylene containers at room temperature. Test solutions were prepared and adjusted to pH 6.5 with 0.1 N HCl or 0.1 N KOH at the time of testing.

Test Organisms. Seeds from five plant species were used in this test: Lettuce (Lactuca sativa L. var. Buttercrunch), radish (Raphanus sativus L. var. Cherry Belle), wheat (Triticum aestivum L. var. Stephens), cucumber (Cucumis sativus L. var. Hybrid Spartan Valor), and red clover (Trifolium pratense L. var. Kenland). Lettuce and cucumber seeds were obtained from George W. Park Seed Company and radish seeds were purchased from Northrup King Seed Company. Wheat and red clover seeds were obtained from the Oregon Seed Certification Office and Corvallis Feed and Seed, Inc., respectively. The seeds used in all laboratories were from the same untreated (no fungicides or repellants) seed lots. They were separated by size with a series of screens and only one size was used. Seeds were stored in closed containers at 5°C until use.

Facilities and Equipment. When testing potentially hazardous substances, solution preparation, planting and root measurements were done in a fume hood. A controlled environment chamber was used to maintain a uniform testing temperature at 25 ± 1°C. One-piece molded glass tanks (6.5 l capacity, approximately 24.5 cm (L) x 16 cm (W) x 17.5 cm (H)) were used as test containers. Single-strength glass plates (13 cm x 15 cm) were used to support the seed substrate. The plates were held at a 67° angle by glass pegs (2-3 cm long and 5 mm in diameter) cemented to the insides of the glass tank with epoxy cement (Duro-E-Pox-E® resin and hardener, EPF-39) or with a glass rack (Fig. 1).

Prior to testing, the tanks and glass plates were thoroughly washed in warm water with a synthetic detergent (e.g., Alconox). Following washing, the glassware was rinsed in tap water, in 50% nitric acid, and finally with distilled water.

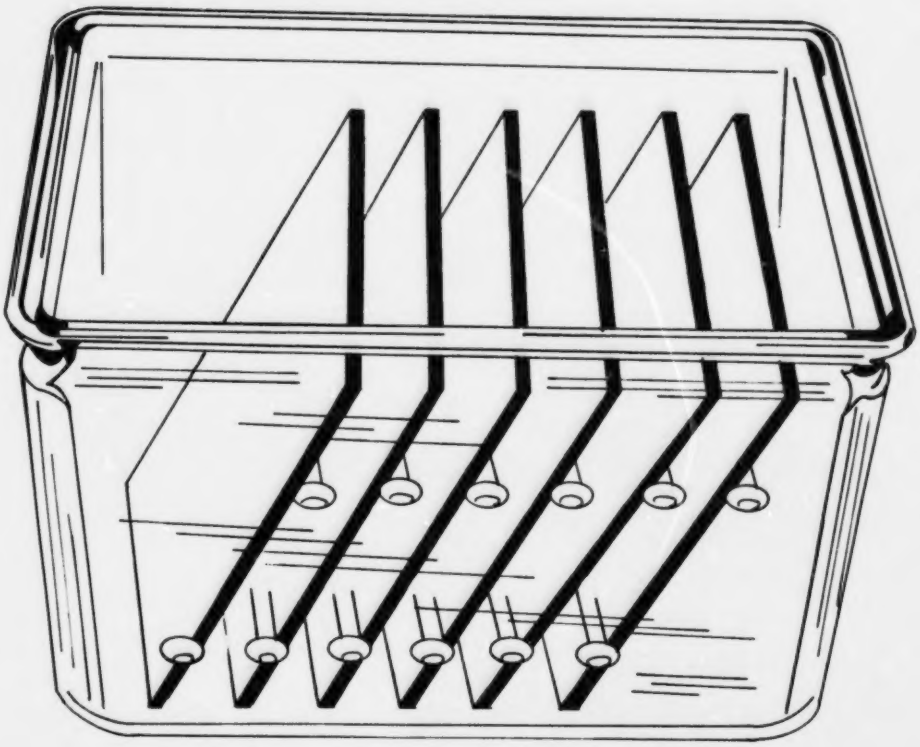

Figure 1. Glass tank with glass plates and filter paper substrate in position between pegs.

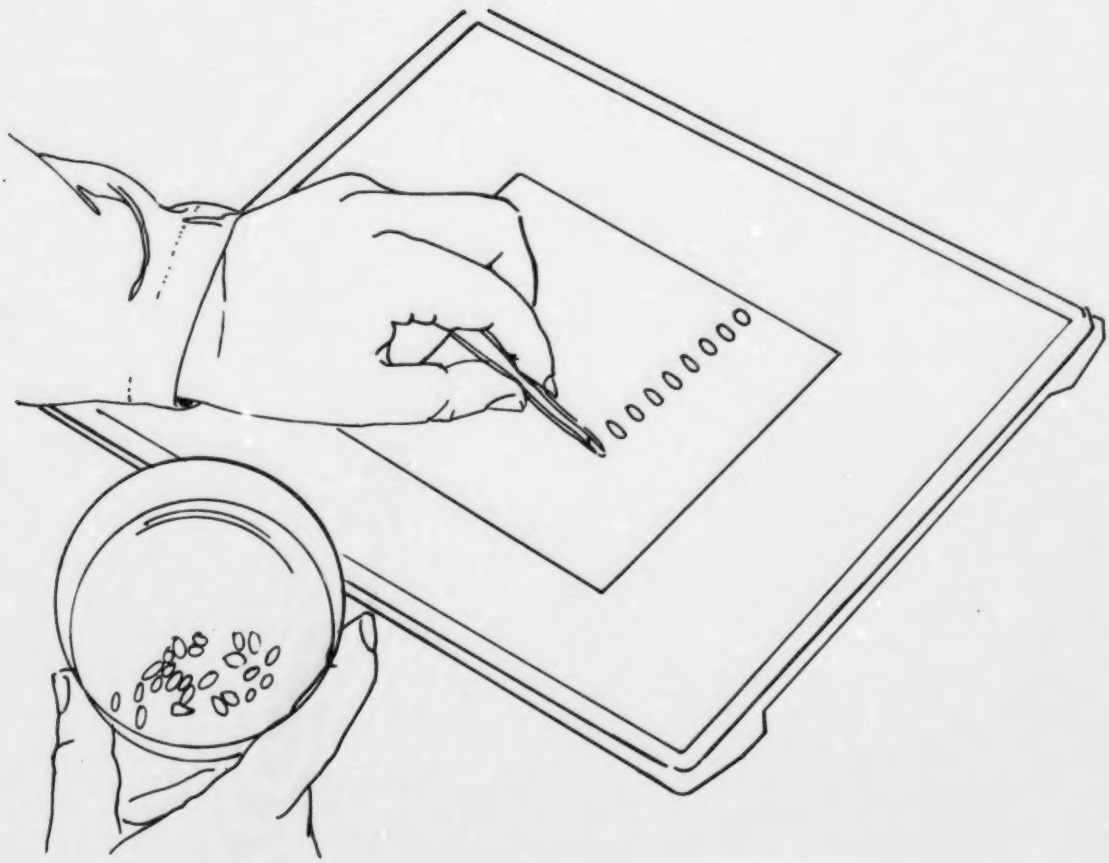

Figure 2. Planting seed on the filter paper substrate.

Procedure for Planting Seed. Whatman 3 MM chromatography filter paper rectangles (13 x 15 cm) were used as the substrate for the seeds. They were saturated in the test solution for a minimum of 5 minutes. One sheet of filter paper was removed from the test solution, allowed to drain, and placed on a glass plate to which the paper adhered. Using forceps, 15 seeds from one species were placed with equal spacing 2 cm down from the top edge of the filter paper (Fig. 2). The radicle end of each seed was oriented toward the bottom of the plate, and in the case of wheat the embryo side of the seed was up. The row of seeds were held in place by a narrow strip of clean tissue (Kimwipes®) placed over them. With the larger seeds, it was necessary to use a fine distilled water mist to moisten the tissue and thus secure the seed to the filter paper. These tissues were previously extracted with distilled water for a minimum of 24 hours in a soxhlet extractor. After extraction, the tissues were removed, air dried, and stored in a dry container or plastic bag. For use, the tissues were cut into narrow (1-2 cm wide) strips. One liter of test solution was poured into the glass tank. The glass plate holding the seeds and filter paper was inserted in the tank between the peg guides to support the plates at an angle of 67° from horizontal. The bottom 2 cm of the plate was immersed in the test solution. This procedure used five plates (one species per plate) per tank. In the same manner, a tank was prepared for each test solution (at least three concentrations). Two distilled water control tanks were also prepared for each experimental set.

Incubation. The glass tanks containing seeds and test solutions were enclosed in heavy polyethylene bags and tied shut to prevent evaporation. They were placed in temperature controlled chambers in the dark and maintained at 25 ± 1°C.

Measurement of Root Length. Root length was measured after 115 ± 0.5 hours from the start of dark incubation. The tanks were uncovered, and each plate was removed and placed on a flat surface (Fig. 3). Radish, lettuce, cucumber, and red clover root lengths were measured from the transition point between the hypocotyl and root to the tip of the root (Fig. 4). For wheat, the single longest primary or seminal root was measured from the point of seed attachment to the root tip. Photographs of seedlings for each species were provided to define the transition points and point of seed attachment. The lengths of all roots were measured to the nearest mm and recorded on data sheets.

Seed Germination and Seedling Morphology. Simultaneously with root measurement, germination and seedling morphology data were recorded. Germination was defined as the emergence of plant structures from the seed with sufficient development for measurement. Seedling morphology was evaluated by selecting a category that best described the morphology of treated seedlings from a list of morphological descriptions.

Testing Protocol. Tests were performed by exposing seed from all five species to concentrations of the test chemical. The first test included a wide range of concentrations to determine whether the test chemical was phytotoxic and to find the appropriate sensitivity range for each species. Subsequent tests included chemical concentrations selected to cover the sensitivity range previously estimated. The chemical concentrations selected were generally equally spaced on a log scale.

The objective of the testing was to estimate the concentration of chemical which reduced root length by 50% of the control length. To ensure

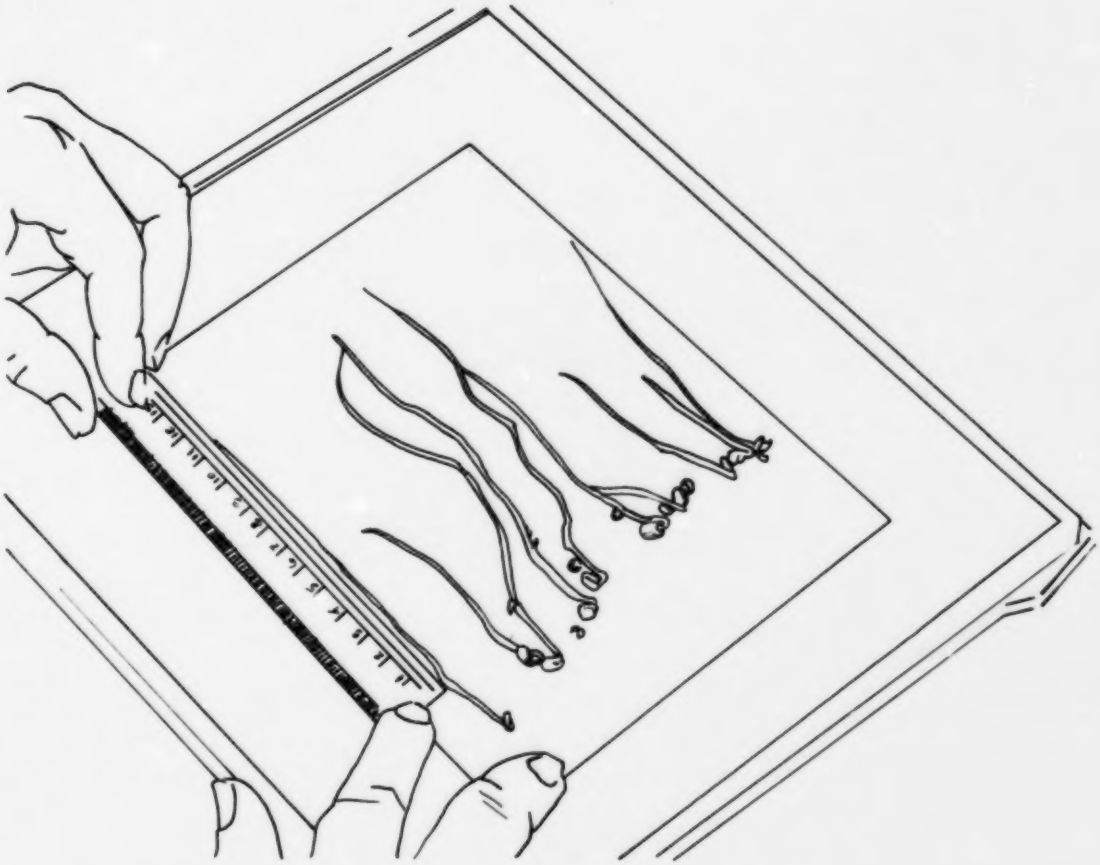

Figure 3. Root length measurement of radish seedlings.

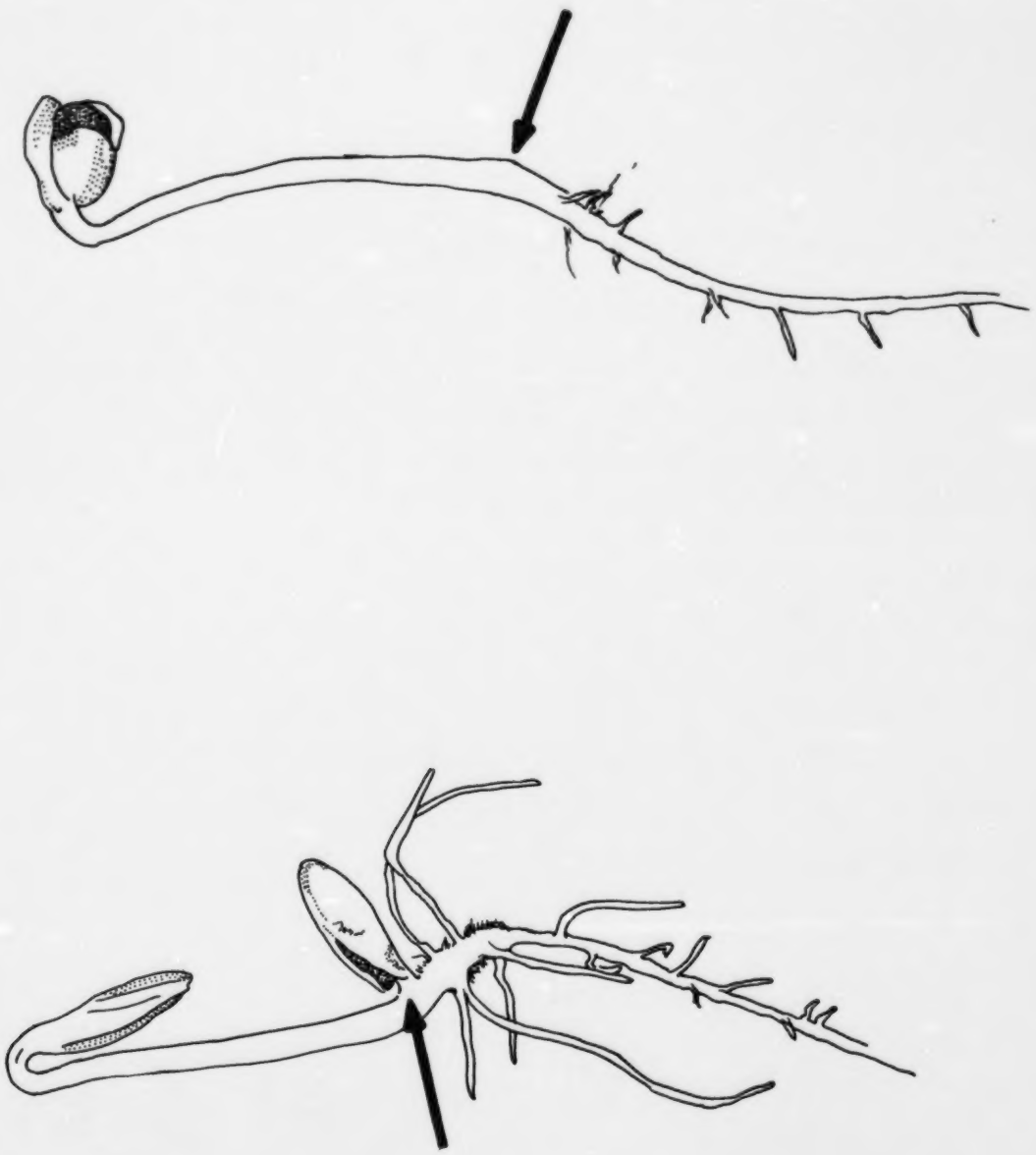

Figure 4. Radish (above) and cucumber (below) seedlings with pointer showing transition point between root and hypocotyl.

sufficient data for an estimate, the following testing criteria were established:

Either:

1. For each species, at least one test must include three concentrations in which mean root length was between 12-85% of the controls, with one greater than 50% of control, and one smaller than 50%, and the third within 12-85%.

Or:

2. For each species, at least one test must include one concentration in which mean root length was between 60-80% of control and a second concentration in which mean root length was between 15-40% of control.

If mean root length was based on the germination of fewer than 10 seeds per plate for controls or test concentrations, that data was not used to satisfy criterion 1 or 2.

Statistical Methods. Test data for each laboratory and chemical were used to describe dose-response curves in which root length was plotted against the log 10 of the test concentration. Only data from the linear portion of the dose-response curves were selected to calculate an EC50. The EC50 is defined in this bioassay as the chemical dose (mg/l) required to reduce root length of treated seedlings to 50% of control. The regression of y(mean root length) on x (log 10 dose) was calculated from the selected data and the

equation was used to calculate the estimated dose required to produce a root length of ½ of the control. The variances of the control and treated root lengths and the dose were used to compute a standard error of the EC50 estimate.

RESULTS

Control Root Length

In quantitative growth-inhibition assays minimum root length is zero, but the maximum length is expressed in relation to the mean length of the control seedlings, and the dose required to reduce the root length by 50% can be estimated (EC50). Measurement inaccuracies and biological variability contribute to the error in the estimated maximum length. This and similar errors with treated seedlings reduce the precision of the EC50 estimate. Therefore, it is important to examine the variation in control root lengths for each laboratory.

The control root lengths for each species at each laboratory are shown in Table I. The mean root length ($\bar{x}$) represents 15 roots of each species measured in each of (N) number of control tests. The variation or standard deviation (s) in mean root length within laboratories results from seedling variability, the variation in environmental controls and the experimental error of the testing laboratory.

For laboratory 5, the means for each species, except wheat, are smaller than means for other laboratories. Means for wheat, cucumber and radish from laboratory 3 are larger than other laboratories and this is reflected in the mean value of all species of 91 mm. The standard deviation of the mean tends to increase as the species root lengths increase, but within species the standard deviations are relatively consistent for laboratories. For example,

Table I. Control mean root length ($\bar{x}$) in mm, standard deviation (s), and coefficient of variation ($\bar{V}$) for laboratories and species.

	Red Clover				Lettuce				Wheat				Cucumber				Radish				
Laboratory	N	$\bar{x}$	s	$\bar{V}$	N	$\bar{x}$	s	$\bar{V}$	N	$\bar{x}$	s	$\bar{V}$	N	$\bar{x}$	s	$\bar{V}$	N	$\bar{x}$	s	$\bar{V}$	$\bar{x}$
1	63	25	4	17	64	69	5	7	64	80	8	10	64	103	12	12	64	129	29	23	82
2	44	23	3	14	35	60	6	10	44	66	12	18	44	111	10	9	44	134	24	18	79
3	50	27	5	20	49	62	8	12	50	91	12	13	50	118	16	13	50	156	29	19	91
4	54	25	6	24	54	59	12	20	54	76	17	22	54	112	15	13	54	138	39	28	82
5	74	22	5	25	76	50	12	23	76	78	11	14	74	87	23	27	75	95	36	37	67
6	56	32	6	17	54	67	7	10	56	64	10	15	60	113	13	12	58	133	28	21	82
7	41	28	4	15	41	64	8	13	41	76	7	10	41	95	19	20	41	126	18	14	78
$\bar{x}$		26				62				76				106				130			

the standard deviation varied from 3-39 among species, but the largest change was from 7-17 within a species.

The coefficient of variation ($\bar{V}$) was computed to show the relative precision for control root lengths. These data indicate an apparent consistency in the amount of within and between species variability. The coefficients of variation do not appear to vary more from species to species than they do within species. However, in 4 out of 7 laboratories the coefficients of variation were largest for radish and in 3 out of 7 laboratories the coefficients of variation were smallest for lettuce.

Because of the homogeneity of the coefficient of variation, a three factor analysis of variance on logs of control means was used to test the effects of laboratory, species and zero dose (chemical). Because of the possibility of diffusion of a volatile chemical through the polyethylene membrane from a treatment to a control, controls by chemical were tested as a third factor. The control mean root lengths by laboratory, species and zero dose (chemical) are shown in Appendix B*, and the analysis of variance is shown in Table II. There is a significant difference in control means between laboratories, which was suspected from examination of Table 1. There is no significant difference in control means between chemicals tested. Statistically there was a significant interaction between laboratory and zero dose (chemical) and between laboratory and species. However, only the laboratory and species interaction was considered large enough to be important. This

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* Control mean root lengths in Appendix B were determined for a single chemical tested, in contrast to control means in Table I which were averaged over eight chemicals tested.

Table II. Analysis of variance table for control mean root length by laboratory, species, and zero dose (chemical) with the laboratory x species x zero dose interaction designated as error.

Source	df	MS	F	P-value
Laboratory	6	0.0622	41.47	2.10
Zero Dose (Chemical)	7	0.0021	1.40	2.01
Species	4	4.0820	2,721.33	2.37
Laboratory x Zero Dose	42	0.0035	2.33	1.38
Laboratory x Species	24	0.0163	10.87	1.52
Zero Dose x Species	28	0.0017	1.13	1.48
Laboratory x Zero Dose x Species	168	0.0015		
Totals	279			

interaction can be seen most clearly by comparing mean root lengths for cucumber and lettuce for laboratories 1 and 7 and laboratories 3 and 4.

Mean Root Length (mm)

Laboratory	Lettuce	Cucumber
1	69	103
7	64	95
mean for all labs	62	106
3	62	118
4	59	112

For laboratories 1 and 7, mean root lengths for lettuce are above the mean for all laboratories, while root lengths for cucumber are below the mean for all laboratories. For laboratories 3 and 4, the reverse is true.

A possible explanation of this interaction could be due to the effect of temperature on root elongation. If deviation of 1 - 2°C above 25°C occurred in some laboratories and 1 - 2°C below 25°C in others the interaction could be explained as follows: The optimum temperatures for cucumber and lettuce growth are 30°C and 25°C, respectively. A temperature above 25°C would thus favor cucumber growth relative to lettuce, and a temperature below 25°C would favor lettuce relative to cucumber. We have not tested this possibility but draw attention to it as a matter of interest. These data which reveal a significant difference in mean root length between laboratories and a significant interaction between laboratories and species emphasize the need to include control treatments with each chemical series and species tested.

Seed Germination and Seedling Morphology

Data including germination results and the categories describing seedling morphology were examined but not analyzed statistically. The laboratory selection of a category that best described morphology was subjective and the

results were useful only when effects appeared to be abnormal. The majority of the observations fell into two categories: either the emerging plant structures were normal in development (only reduced in length) or roots were abnormal with visible necrosis or were weak and spindly. An exception occurred with 2,4-D where the category indicating abnormal development with short, stubby and thickened roots was often selected. Other categories describing the morphology of the seedling were infrequently selected.

The results of the germination tests showed that seed germination was not significantly inhibited at those chemical concentrations in which root growth was inhibited, with the exception of Carbowax and cineole. Germination of lettuce and radish seed was inhibited at nearly the same concentrations of cineole required to severely inhibit root growth. The concentration of carbowax that inhibited root growth was near the threshold for inhibiting seed germination for all species, except cucumber.

Dose-Response

The dose-response relationship provides a basis for estimating the phytotoxicity of a test chemical. The measured response (root length) of the treated seedlings depends on the dose (chemical concentration). Figure 5 shows a typical dose-response curve for test data in which root length was plotted against the log 10 of the concentration of the treatment. Data in the linear portion of the curve where small changes in dose resulted in large changes in root length best describe the dose-response relationship. To select data in the linear range of doses for the final analysis, treated mean root length as percent of control mean root length was plotted against log 10 dose (mg/l) for each chemical, species and laboratory. A straight line was fitted by eye to the middle portion of the curve emphasizing the points in the

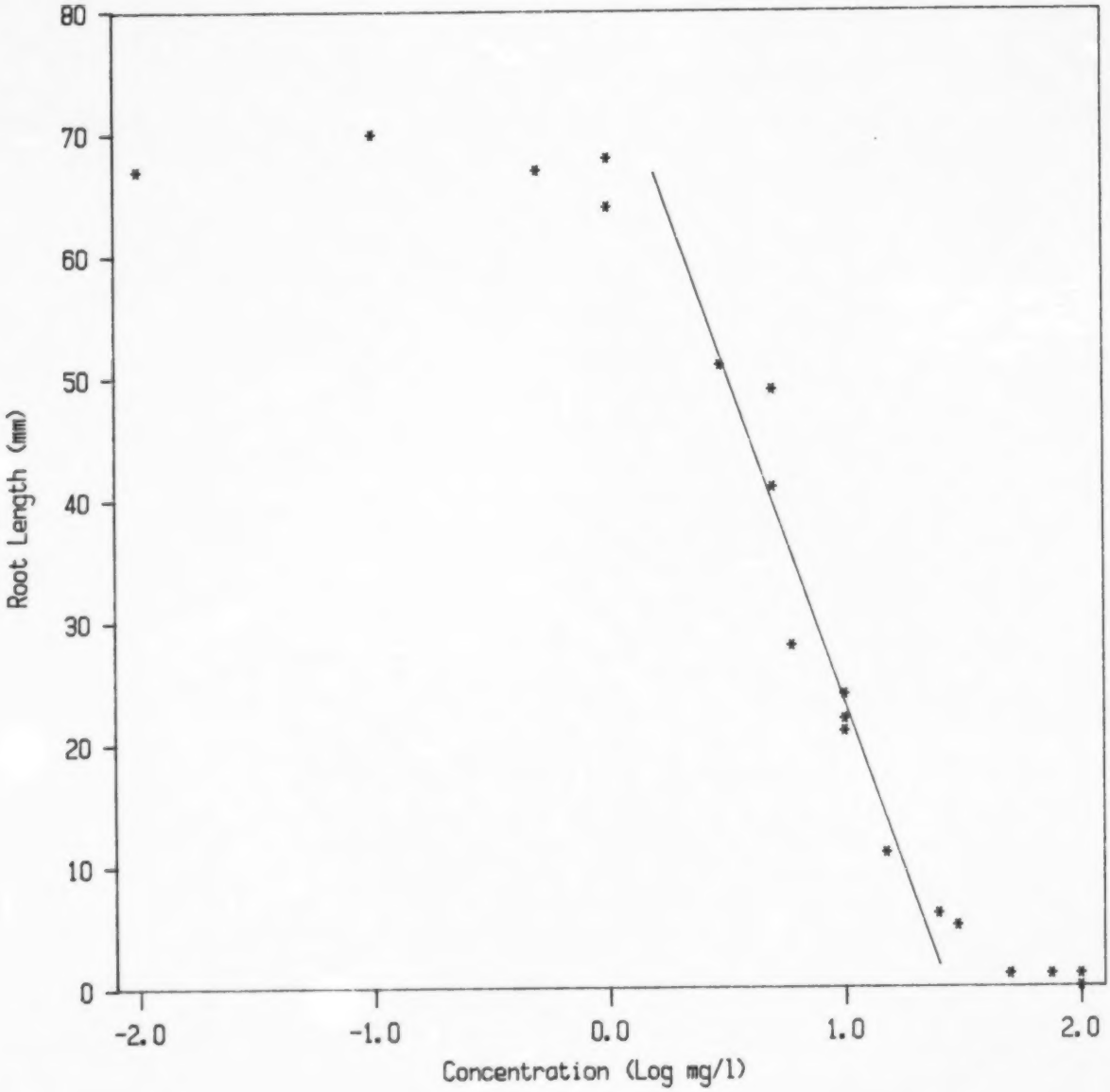

Figure 5. Dose-response curve for lettuce and cadmium chloride using data from laboratory no. 1.

20-80% of control mean range. The line was extended to intersect both the 100% and 0% of control lines, and these intersections and the corresponding points on the log dose axis were designated as upper and lower dose limits. Only data from doses within the upper and lower limits were used in subsequent regression analysis.

The toxicity parameter (EC50) in this assay is defined as the chemical dose (mg/l) required to reduce root length of treated seedlings to 50% of control. Data sets consisting of log 10 dose (mg/l) and mean root length (mm) within the established dose limits were selected for each laboratory, chemical and species. These data were used to compute an EC50 by calculating the regression of y (mean root length) on x (log 10 dose) and calculating $\hat{x}$ for 1/2 the mean control length (see Appendix C).

RESULTS: TOXICITY TESTS

EC50 values (mg/l) and multiplicative standard error (see Appendix C) were calculated from the toxicity test data from each of the laboratories and are listed in Table III. Results for endosulfan and pentachloronitrobenzene (PCNB) are not included since saturated solutions of these chemicals caused no significant inhibition of root growth for any species at any of the participating laboratories. All toxicity test data from the laboratories were included except: 1) data from tests that were indicated as invalid by the testing laboratory because of suspected errors in chemical preparation or reported equipment malfunctions, 2) when fewer than 67% of the seed germinated on a test plate, and 3) data outside the upper and lower dose limits that were established for each chemical, species and laboratory.

The EC50 values in Table III range from 5×10^{-3} mg/l for 2,4-D to 366×10^{3} mg/l for carbowax, indicating test species sensitivity over a wide range

Table III. Calculated EC50 values and standard error by chemical, species, and laboratory.

	Red Clover		Lettuce		Wheat		Cucumber		Radish	
Laboratory	EC50	SE	EC50	SE	EC50	SE	EC50	SE	EC50	SE
Silver Nitrate, mg/l										
1	237	3.98	1	1.52	99	1.28	8	1.34	38	1.56
2	275	1.50	8	1.53	321	1.47	32	1.33	77	1.39
3	101	1.26	3	1.46	161	1.33	9	2.32	76	1.29
4	56	1.28	5	2.61	173	1.81	24	1.53	84	1.98
5	119	1.20	1	2.54	103	1.82	72	1.89	134	1.58
6	53	1.30	4	1.57	172	1.21	42	1.45	85	1.44
7	32	1.33	1	2.82	145	1.52	5	2.10	23	2.81
Sodium Fluoride, mg/l										
1	368	1.06	463	1.07	225	1.18	167	1.32	366	1.09
2	217	1.12	346	1.11	261	1.26	139	1.15	254	1.06
3	425	1.11	573	1.14	179	1.12	140	1.13	286	1.18
4	273	1.25	464	1.12	232	1.23	174	1.14	361	1.17
5	224	1.13	629	1.41	224	1.21	154	1.15	534	1.56
6	353	1.12	475	1.07	260	1.19	174	1.19	373	1.08
7	321	1.31	527	1.21	286	1.35	217	1.54	306	1.22
Cadmium Chloride, mg/l										
1	37	1.25	6	1.09	66	1.24	10	1.23	13	1.55
2	27	1.28	10	1.17	81	1.51	32	1.12	18	1.18
3	13	1.33	6	1.35	75	1.33	15	1.16	12	1.45
4	10	1.63	15	1.28	145	1.38	26	1.47	21	1.71
5	7	1.82	2	1.74	90	1.84	7	1.57	7	1.91
6	12	1.20	6	1.16	56	1.24	23	1.25	33	1.25
7	23	1.45	15	1.18	185	1.28	25	1.31	28	1.20
MAA, mg/l										
1	21	1.55	30	1.33	962	1.21	116	1.10	19	1.41
2	15	1.55	8	*(a)	464	1.50	77	1.18	15	1.78
3	21	2.17	19	2.06	608	1.48	43	1.25	21	1.46
4	15	1.53	36	1.99	855	1.18	93	1.12	17	1.34
5	27	1.37	15	2.06	480	1.32	121	1.12	36	5.20
6	24	1.63	18	2.31	855	1.12	133	1.19	61	1.57
7	9	1.80	16	1.57	545	1.22	67	1.18	32	1.17

(continued)

(a) Variance component (s^2 pooled) of the standard error could not be calculated with only one valid control mean.

Table III (continued)

Laboratory	Red Clover EC50	Red Clover SE	Lettuce EC50	Lettuce SE	Wheat EC50	Wheat SE	Cucumber EC50	Cucumber SE	Radish EC50	Radish SE
Monuron, mg/l										
1	57	1.51	78	1.11	147	1.38	69	1.09	88	1.25
2	53	1.31	43	1.17	172	1.19	65	1.10	75	1.30
3	82	1.67	84	1.54	148	1.25	61	1.63	57	1.48
4	125	1.55	151	1.36	*	*(b)	186	1.16	206	1.28
5	57	1.65	77	1.37	170	1.17	106	1.33	114	1.24
6	39	1.46	72	1.17	167	1.22	64	1.18	69	1.28
7	39	1.40	79	1.25	273	1.35	78	1.15	88	1.41
2,4-D, mg/l x 10^{-3}										
1	37	1.35	35	1.12	914	1.43	19	1.12	60	1.17
2	66	1.17	55	1.25	5773	1.54	22	1.28	50	1.26
3	128	2.87	68	2.23	753	1.64	30	1.66	34	1.43
4	42	2.78	58	1.46	1851	1.94	24	1.57	18	3.30
5	30	1.47	17	1.87	10274	4.51	17	1.99	27	1.42
6	8	4.58	5	3.82	1880	1.39	160	1.24	27	1.95
7	61	1.35	66	1.47	4785	1.52	45	1.16	51	1.31
Carbowax, mg/l x 10^{3}										
1	*(b)	693.00	264	1.08	197	1.15	124	1.14	201	1.21
2	*(e)	*	*	*(c)	147	1.55	52	1.34	220	1.41
3	188	1.22	179	1.26	195	1.07	75	1.25	68	1.58
4	153	*(d)	136	1.71	197	*(d)	55	1.32	45	2.13
5	*(b)	28.94	320	2.53	366	2.19	78	1.49	*	*(b)
6	117	1.09	115	1.24	207	1.13	54	1.27	77	1.18
7	179	1.39	42	1.26	191	1.11	105	1.28	90	1.22
Cineole, mg/l										
1	1175	1.24	*	*(b)	375	1.12	666	1.07	514	1.23
2	2787	1.29	680	1.55	1342	1.79	4937	1.70	1310	1.19
3	4179	2.74	725	*(d)	1851	2.41	1602	1.15	*	*(b)
4	2290	1.16	637	1.42	860	1.42	1414	1.10	1325	1.18
5	901	1.72	*	*(c)	1184	1.19	1117	1.75	*	*(b)
6	2664	1.27	841	9.78	1058	1.58	1894	1.19	1342	2.19
7	2900	1.14	*	*(c)	1754	1.25	2548	1.06	2622	1.39

(b) Standard error term extremely large and EC50 estimate considered invalid.

(c) EC50 and standard error could not be calculated with only one valid treatment dose.

(d) Variance component ($s^2_{y/x}$) of the standard error could not be calculated with only two valid treatment doses.

(e) EC50 and SE invalid, slope (β) of the regression inconsistent with other data.

of concentrations of different chemicals. For some chemicals there are large differences in species sensitivity, while in others the EC50 estimates do not differ greatly. For example, EC50's for MAA range from 464 - 962 mg/l for wheat and from 9 - 27 mg/l for red clover. For sodium fluoride, the EC50's for wheat and red clover range from 179-286 mg/l and 217-425 mg/l, respectively. Estimating an EC50 for cineole and Carbowax was more difficult than for the other chemicals, since germination and root elongation were inhibited at nearly the same levels. Those chemical concentrations required to inhibit root length below 50% of control length also inhibited germination, resulting in insufficient data to estimate an EC50. Those concentrations where fewer than 10 seeds germinated were not included in the data, and the criteria for estimating an EC50 could not be met.

An analysis of variance of the log 10 of the estimated EC50's was calculated for laboratory, chemical and species (Table IV). Transformed data were used because variances were not homogeneous from chemical to chemical. Carbowax and cineole were not included in the analysis because of the large number of missing values. The mean of the EC50 estimates for the other six laboratories was substituted for the missing value in laboratory 4 for the effect of monuron on wheat.

The analysis of variance shows that the estimated EC50's for six chemicals and for five species were significantly different (Table IV). The differences due to chemical are considerably larger than those for species. There are no significant differences among the laboratory estimated EC50's for chemicals and species. This implies that all laboratories were equally accurate in estimating EC50's for each of the six chemicals and five species. The laboratory x chemical and laboratory x species interactions are statistically significant but are relatively small compared to the other mean squares.

Table IV. Analysis of variance table for log 10 EC50's by chemical, species, and laboratory with the laboratory x chemical x species interaction designated as error.

Source	df	MS	F	P-value
Laboratory	6	0.04	1.14	2.17
Chemical	5	53.83	1,376.64	2.29
Species	4	6.70	171.38	2.45
Laboratory x Chemical	30	0.12	3.19	1.55
Laboratory x Species	24	0.08	2.15	1.61
Chemical x Species	20	1.27	32.48	1.66
Lab x Chemical x Species	120	0.04		
Totals	209			

The chemical x species interaction is significant and is proportionally much larger than laboratory x chemical or laboratory x species interaction.

Since there were no statistically significant differences among laboratories, overall EC50 means were calculated for all laboratories to compare chemicals and species (Table V). Wheat is consistently the most tolerant species (largest EC50) for the chemicals tested except sodium fluoride. Lettuce and red clover were generally the most sensitive (smallest EC50), although for sodium fluoride, lettuce had the largest EC50.

By comparing the responses of different species to the chemicals tested, the nature of the toxicity of the chemicals can be determined. Sodium fluoride was unique because all the species tested were considerably more tolerant to it than the other five test chemicals. Also the responses for all species occurred in a similar concentration range of sodium fluoride. It also exemplifies the chemical x species interaction in which wheat, generally more tolerant, was more sensitive than lettuce, which was generally more sensitive. These results indicate a non-selective mode of action for sodium fluoride in inhibiting root growth.

Silver nitrate and cadmium chloride have similar species response patterns in which lettuce is the most sensitive species tested and wheat is the most tolerant. The range of mean EC50's for both chemicals (3 to 156 for silver nitrate and 7 to 92 for cadmium chloride) is large compared to other chemicals tested. These results indicate a more selective mode of action in which chemicals inhibit root growth to a greater degree in some species than in others.

For monuron, MAA and 2,4-D, red clover is the most sensitive species, and wheat is the most tolerant. However, for monuron and 2,4-D, the differ-

Table V. EC50 (mg/l) geometric means for all laboratories for chemical and species†.

Species	$AgNO_3$	$CdCl_2$	Monuron	MAA	NaF	2,4-D	PCNB	Carbowax	Cineole	Endosulfan
Cucumber	19 (4)	18 (2)	83 (3)	87 (2)	165 (5)	0.03 (2)	*	74,000(5)	1695(2)	*
Lettuce	3 (5)	7 (5)	79 (4)	18 (4)	489 (1)	0.03 (3)	*	$147{,}000_c$(3)	717_a(5)	*
Radish	65 (3)	17 (3)	91 (2)	25 (3)	345 (2)	0.04 (4)	*	$99{,}000_c$(4)	1257_b(3)	*
Red Clover	96 (2)	16 (4)	60 (5)	18 (5)	303 (3)	0.04 (5)	*	$157{,}000_a$(2)	2158(1)	*
Wheat	156 (1)	92 (1)	176 (1)	655 (1)	236 (4)	2.54 (1)	*	207,000(1)	1084(4)	*

(†) Species ranks within chemical where 1 is most tolerant and 5 is most sensitive are in parentheses.
* No significant inhibition of root growth.
(a) Geometric mean based on estimates from 4 laboratories.
(b) Geometric mean based on estimates from 5 laboratories.
(c) Geometric mean based on estimates from 6 laboratories.

ences in the mean EC50's are small if wheat is excluded. The EC50 means for cucumber, lettuce and radish vary more for MAA than in monuron and 2,4-D.

As previously discussed, the six test chemicals can be arbitrarily placed into three groups based on differential species response. This grouping corresponds to a general chemical classification in which sodium fluoride represents an inorganic anion (F^-), silver nitrate and cadmium chloride represent inorganic cations (Ag^+, Cd^{2+}), and monuron, MAA and 2,4-D represent organics and organometallics. Since these observations are based on a small number of chemicals, it is questionable whether a similar classification could be applied to a large number of diverse chemicals. The value of using several species in testing a chemical is that the differences or similarities in responses may be used to characterize a general type of toxic action for that chemical. In hazard assessment, non-selective chemicals may require little additional testing, while selective chemicals may require testing with other species of specific importance to the region of impact.

To determine the precision of the EC50 estimate of the testing laboratories, an analysis of variance was calculated for the standard error by laboratory, chemical and species. Table VI shows that the standard errors in estimating the EC50 are significantly different among chemicals tested, which is the largest mean square error. A general property of the dose-response curve is that error in the EC50 estimate is related to the slope of the regression line; a steep slope indicates a relatively small error and a shallow slope indicates a larger error. Since it is generally accepted that the slope of the response curve differs depending on chemical, it is expected that the standard error would also differ with chemical. In Table VII standard errors averaged over species are relatively small for sodium fluoride and relatively large for silver nitrate. An examination of the slope of the

Table VI. Analysis of variance table for standard error by chemical, species, and laboratory.

Source	df	MS	F	P-value
Laboratory	6	0.04	4.30	2.17
Chemical	5	0.13	14.38	2.29
Species	4	0.03	3.14	2.45
Laboratory x Chemical	30	0.02	1.86	1.55
Laboratory x Species	24	0.01	1.00	1.61
Chemical x Species	20	0.02	1.76	1.66
Lab x Chemical x Species	120	0.01		
Totals	209			

Table VII. Standard error means (Log 10) for all species for laboratory and chemical.

Laboratory	$AgNO_3$	$CdCl_2$	Monuron	MAA	NaF	2,4-D
1	0.2428	0.1021	0.0994	0.1179	0.0572	0.0908
2	0.1599	0.0947	0.0828	0.1922	0.0563	0.1122
3	0.1727	0.1200	0.1785	0.2166	0.0546	0.2797
4	0.2526	0.1725	0.1192	0.1464	0.0727	0.3221
5	0.2439	0.2491	0.1276	0.2675	0.1079	0.3084
6	0.1420	0.0867	0.0991	0.1796	0.0525	0.3532
7	0.3058	0.1077	0.1176	0.1354	0.1215	0.1320

regression lines used to calculate the EC50 indicates greater slopes for sodium fluoride than for silver nitrate. For example, the mean slopes for all laboratories for lettuce and radish are -68 and -203, respectively, for sodium fluoride and -17 and -56, respectively, for silver nitrate.

As shown in Table VI, there is a significant difference in standard error of the EC50 estimate for laboratories. From this data it is concluded that although the laboratories were able to accurately estimate an EC50, the variability of the method was sufficiently large so that the precision of the estimate differs significantly.

The standard error of the EC50 estimates are also significantly different for species. Cucumber tended to have the smallest standard error means for all laboratories, while values for other species were not appreciably different.

The interactions between laboratory, chemical and species do not appear to be an important factor, although the laboratory x chemical and chemical x species interactions are statistically significant.

The precision in which the EC50 is estimated by a laboratory depends on the following: 1) how well the doses were selected in terms of equal spacing on a log scale and the number of doses on the linear part of the response curve, 2) the slope of the regression line, 3) the accuracy of the preparation of the chemical dose, and 4) the variability of the root length response for each species. The critical factor appears to be the allocation of an adequate number of chemical concentrations which give responses in the linear portion of the dose response curve.

CONCLUSIONS

Reduction in root length is a valid and sensitive plant response to exposure to chemical substances. This is a suitable test for evaluating phytotoxic substances over a wide range of concentrations.

The interlaboratory testing procedures of this bioassay provided a uniform root growth environment. The results within and among laboratories showed this uniformity. Most variation was due to biological differences among species.

Using a single method to evaluate five species simultaneously was cumbersome. It is difficult to satisfy all criteria for all the species at the same time. The precision of the EC50 estimates varied significantly indicating the need for more data points to adequately define the dose-response relationships for all five species. A minimum of four observations on the linear portion of the doseresponse curve should be used. These points should alos be equally spaced on a log scale with the interval between doses approximately 1.5 to 2.0.

Although this test is difficult to use on five species at once, it might be used more efficiently for one species at a time. The results from a single species test would give some indication of the general response of most species but would not reflect the response of all species.

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Appendix A

Participating Laboratories

1. Corvallis Environmental Research Laboratory
   Environmental Protection Agency
   Corvallis, Oregon

2. Northwest Biological Investigation Station
   Environmental Protection Agency
   Corvallis, Oregon

3. Environmental Monitoring and Systems Laboratory
   Environmental Protection Agency
   Las Vegas, Nevada

4. Crop Science Department
   Oregon State University
   Corvallis, Oregon

5. Statewide Air Pollution Research Center
   University of California
   Riverside, California

6. Northrop Services, Inc.
   Little Rock, Arkansas

7. Chinook Research Laboratories, Inc.
   Corvallis, Oregon

Appendix B. Species and Laboratory Mean Control Mean Root Length (mm) Calculated When Testing the Indicated Chemical

	Red Clover			Lettuce			Wheat			Cucumber			Radish		
	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N
Laboratory															
Silver Nitrate															
1	25.20	3.71	10	69.20	5.41	10	77.60	8.76	10	98.00	14.28	10	122.70	28.34	10
2	22.00	4.40	4	61.67	7.77	3	61.75	9.03	4	113.25	7.74	4	111.50	10.76	4
3	30.00	3.92	4	59.50	2.52	4	93.25	4.03	4	128.25	20.66	4	157.25	21.33	4
4	30.75	4.03	4	67.50	6.24	4	66.00	13.14	4	117.00	5.48	4	124.50	36.24	4
5	23.60	3.36	5	52.67	6.89	6	88.67	3.14	6	61.17	17.54	6	73.00	20.93	6
6	32.33	5.82	6	70.17	7.25	6	64.33	3.39	6	118.17	9.60	6	146.33	33.48	6
7	26.67	3.51	3	61.67	8.50	3	80.33	11.85	3	101.33	13.87	3	125.33	38.02	3
$\bar{x}$	27.22			63.20			75.99			105.31			122.90		
s	3.89			6.22			12.37			22.02			26.95		
Sodium Fluoride															
1	24.54	3.47	11	69.08	7.56	12	80.08	8.65	12	101.42	16.42	12	115.67	31.13	12
2	23.80	3.90	5	55.33	7.02	3	71.80	4.32	5	122.40	13.16	5	129.40	8.74	5
3	28.25	2.25	8	64.00	4.14	8	96.75	5.16	8	130.00	8.25	8	163.37	36.10	8
4	25.20	5.81	10	61.80	9.57	10	74.00	10.97	10	116.00	13.32	10	130.80	40.26	10
5	22.11	5.11	18	48.17	9.01	18	81.94	10.45	18	88.94	21.14	18	89.83	27.71	18
6	35.58	4.64	12	66.83	10.07	12	65.42	12.44	12	112.25	18.36	12	124.00	29.27	12
7	26.50	3.51	6	58.17	4.36	6	81.17	6.31	6	71.50	19.48	6	129.33	13.37	6
$\bar{x}$	26.57			60.48			78.74			106.07			126.06		
s	4.43			7.21			9.92			20.36			21.84		
Cadmium Chloride															
1	28.00	3.55	8	67.75	2.92	8	78.88	7.40	8	97.38	15.33	8	121.12	38.50	8
2	22.00	2.71	7	62.83	5.42	6	76.71	12.50	7	111.14	8.32	7	147.43	20.15	7
3	29.83	3.66	6	62.83	11.57	6	91.17	1.47	6	121.00	11.58	6	160.17	27.92	6
4	25.00	2.28	6	53.00	4.94	6	74.17	10.53	6	112.83	10.87	6	123.33	30.94	6
5	20.80	5.57	10	52.20	11.47	10	74.00	17.27	10	84.00	22.05	10	95.90	42.88	10
6	34.30	5.64	10	67.50	3.14	10	61.70	11.16	10	109.30	8.56	10	124.10	28.28	10
7	26.17	3.97	6	57.83	6.62	6	78.00	6.81	6	84.00	19.47	6	126.67	20.33	6
$\bar{x}$	26.59			60.56			76.37			102.81			128.39		
s	4.64			6.39			8.70			14.61			20.52		
MAA															
1	22.00	3.59	10	68.20	6.12	10	81.20	6.12	10	110.00	5.96	10	149.80	22.44	10
2	22.00	3.65	4	57.00	*	1	65.25	17.44	4	110.50	9.33	4	140.75	30.21	4
3	27.83	5.71	6	61.33	4.72	6	90.83	10.65	6	121.50	11.74	6	153.17	16.50	6
4	24.25	3.33	8	57.00	8.21	8	72.25	8.17	8	118.62	5.07	8	174.75	16.94	8
5	25.67	5.09	6	57.17	12.81	6	78.83	11.23	6	94.67	15.83	6	105.33	42.84	6
6	30.75	4.35	4	65.75	7.27	4	59.75	6.55	4	116.00	5.35	4	124.25	33.60	4
7	31.50	1.92	4	73.00	3.46	4	72.75	3.95	4	107.25	6.24	4	121.25	6.13	4
$\bar{x}$	26.29			62.78			74.41			111.22			138.47		
s	3.89			6.36			10.34			8.90			23.32		
2,4-D															
1	23.25	4.99	4	72.50	1.73	4	80.50	9.95	4	111.00	5.10	4	120.75	12.71	4
2	25.50	1.05	6	61.20	5.36	5	59.17	10.19	6	108.50	10.84	6	119.33	20.59	6
3	20.83	7.63	6	61.00	8.00	6	90.67	14.58	6	103.50	21.54	6	143.83	33.15	6
4	22.70	9.49	10	52.90	18.73	10	77.80	8.15	10	101.70	26.41	10	125.80	53.66	10
5	19.00	5.63	16	47.56	16.31	16	72.88	6.06	16	93.79	26.63	14	111.00	38.53	16
6	31.12	4.94	8	69.38	4.31	8	65.75	6.84	8	114.67	7.64	12	138.80	29.53	10
7	33.67	1.03	6	69.50	5.01	6	75.17	7.47	6	107.67	6.83	6	124.67	16.40	6
$\bar{x}$	25.15			62.01			74.56			105.83			126.31		
s	5.39			9.25			10.19			6.87			11.40		

(continued)

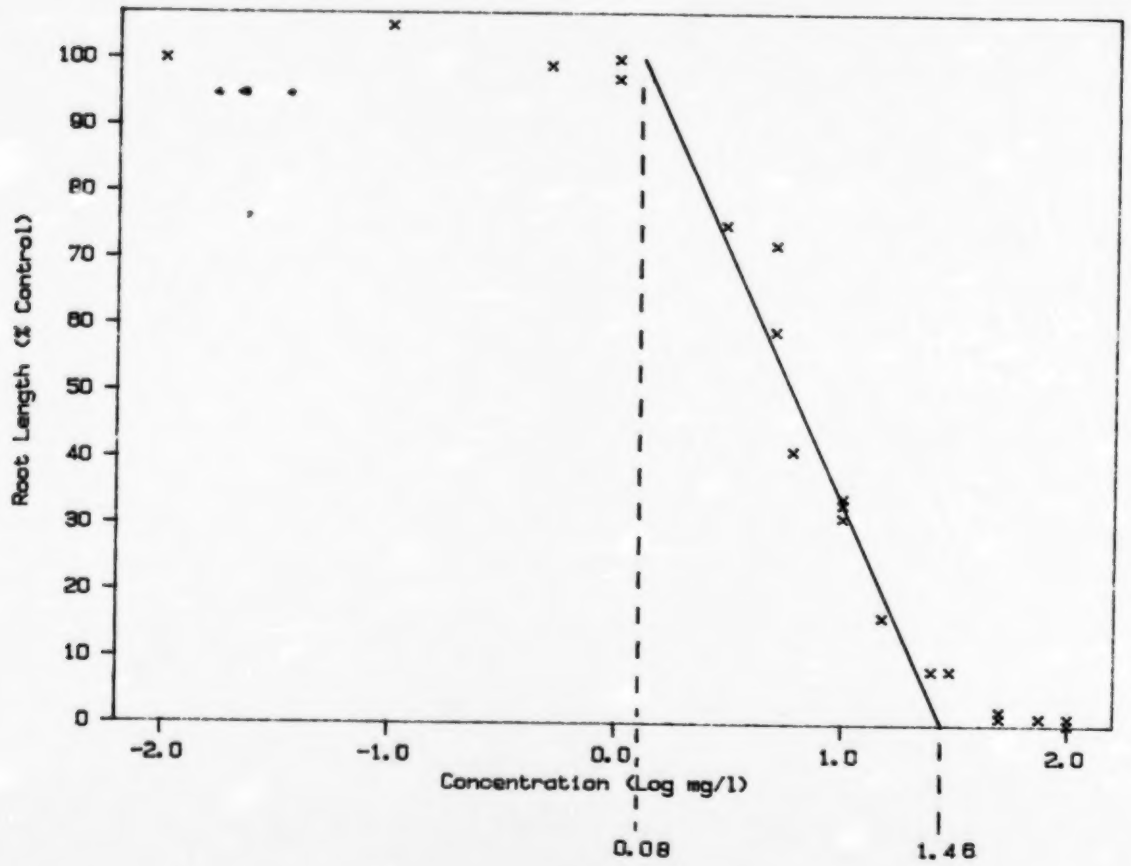
Appendix B (continued)

	Red Clover			Lettuce			Wheat			Cucumber			Radish		
	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N	$\bar{x}$	s	N
Laboratory															
Carbowax															
1	28.25	5.60	8	69.50	4.17	8	76.50	10.42	8	94.25	9.45	8	124.12	32.59	8
2	22.00	3.38	8	57.75	4.77	8	68.25	14.52	8	107.00	9.72	8	130.38	17.98	8
3	25.83	4.71	6	67.50	6.78	6	83.83	7.28	6	120.33	15.67	6	136.50	40.62	6
4	30.33	6.06	6	63.17	8.82	6	89.67	41.72	6	118.17	10.82	6	148.67	42.38	6
5	24.50	5.61	6	51.33	3.78	6	81.50	7.09	6	94.67	25.66	6	118.67	39.40	6
6	28.67	1.75	6	67.67	3.72	6	64.33	5.61	6	119.33	14.43	6	131.17	20.49	6
7	26.75	3.62	8	62.00	9.56	8	77.50	5.83	8	91.00	20.95	8	127.25	17.25	8
$\bar{x}$	26.62			62.70			77.37			106.39			130.96		
s	2.80			6.43			8.79			13.06			9.63		
Monuron															
1	24.67	3.39	6	70.00	3.74	6	86.17	6.46	6	106.67	3.50	6	138.50	23.80	6
2	19.75	1.71	4	61.25	6.85	4	59.25	5.50	4	113.50	4.04	4	135.25	35.50	4
3	27.38	5.10	8	62.29	7.43	7	98.62	16.48	8	112.38	16.58	8	166.25	25.64	8
4	22.83	5.57	6	61.83	10.35	6	77.50	13.79	6	111.50	7.82	6	129.50	28.22	6
5	19.78	3.93	9	46.00	13.01	10	77.20	8.12	10	77.30	23.82	10	66.22	17.03	9
6	29.50	5.45	4	65.00	7.48	4	69.75	7.37	4	118.00	13.59	4	154.75	23.19	4
7	31.75	2.06	4	72.25	2.36	4	71.50	3.32	4	108.50	6.66	4	131.00	14.33	4
$\bar{x}$	25.09			62.66			77.14			106.83			131.64		
s	4.67			8.48			12.58			13.52			31.82		
Cineole															
1	23.00	2.90	6	66.50	3.08	6	86.17	4.45	6	109.00	2.76	6	141.50	13.75	6
2	25.17	2.71	6	62.80	6.38	5	58.83	6.88	6	104.67	8.55	6	153.17	22.91	6
3	30.17	5.27	6	59.00	12.28	6	79.67	11.59	6	112.50	6.22	6	164.17	23.99	6
4	24.00	2.58	4	56.50	8.23	4	75.00	9.02	4	103.75	5.91	4	144.75	7.18	4
5	21.25	4.19	4	59.00	4.16	4	75.75	10.53	4	95.75	14.32	4	107.75	14.98	4
6	25.17	2.64	6	65.00	7.12	4	56.67	12.44	6	101.50	16.96	6	133.50	14.08	6
7	23.50	3.11	4	59.75	5.19	4	64.75	2.63	4	105.00	5.94	4	118.75	24.34	4
$\bar{x}$	24.61			61.22			70.98			104.60			137.65		
s	2.80			3.63			11.08			5.33			19.48		

APPENDIX C

Calculation of the EC50 and Standard Error

1. The test data was plotted (log 10 dose and root length as percent of control) and a straight line was fitted by eye to the middle portion of the curve emphasizing the points in the 20-80% range. The line was extended to intersect both the 100% and 0% of control lines. The intersections and corresponding points of the log 10 dose axis were established as the upper and lower dose limits.



2. Data for EC50 and standard error calculations were selected by using only dose and mean lengths within the established dose limits.

Test	Untreated Control	LOG 10 DOSE (mg/l) -2.0	-1.0	0.0	1.0	2.0
1	66 66	67	70	64	22	0
2	65 71			67 68	49 21	1
3	66 73				41 24 6	1 1 1
4	69 66				51 28 11	5 1 1

$\bar{c}$ = 67.75
s^2_c = 8.50

0.08 lower dose limit

1.46 upper dose limit

LOG 10 DOSE	MEAN LENGTH
0.4771	51
0.6990	49
0.6990	41
0.7782	28
1.0000	21
1.0000	24
1.0000	22
1.1761	11
1.3979	6
$\bar{x}$ 0.9141	$\bar{y}$ 28.11
s^2_x 0.0784	s^2_y 251.61

3. Compute the means and variances for the controls ($\bar{c}$, s^2_c), mean lengths ($\bar{y}$, s^2_y), and dose ($\bar{x}$, s^2_x).

4. Use the selected set of data and compute the EC50 as follows:

   a. calculate the regression of y (mean root length) on x (log 10 dose):

   $\hat{y} = \hat{a} + \hat{b}x = 77.58 - 54.12\ x$

   b. calculate $\hat{x}$ for ½ the mean of controls:

   $$\hat{x} = \frac{(\bar{c}/2) - a}{b} = \frac{67.75/2 - 77.58}{-54.12} = 0.8076$$

   c. EC50 = antilog $\hat{x}$ = 6.42

5. Calculate the "multiplicative standard error" as follows:

   a. calculate the variance, $s^2_{y/x}$ as:

   $$s^2_{y/x} = \frac{n_y - 1}{n_y - 2}\left[s^2_y - \hat{\beta}^2\ s^2_x\right] = \frac{8}{7}\left[251.61 - (54.12)^2\ (0.0784)\right] = 25.298$$

   n_y = number of y's (and x's) used

   b. calculate the variance, s^2 pooled as:

   $$s^2 \text{ pooled} = \frac{(n_c - 1)\ s^2_c + (n_y - 2)\ s^2_{y/x}}{n_c + n_y - 3} + \frac{(7)(8.50) + (7)(25.298)}{8 + 6} = 16.899$$

   n_c = number of c's used

   c. calculate the standard error, SE, as:

   $$SE = \sqrt{s^2}\text{ pooled}\ \frac{1}{\hat{\beta}}\sqrt{\frac{1}{n_c} + \frac{1}{n_y} + \frac{(\hat{x} - \bar{x})^2}{(n_y - 1)s^2_x}}$$

   $$= \sqrt{16.899}\ \frac{1}{54.12}\sqrt{\frac{1}{8} + \frac{1}{9} + \frac{(0.8076 - 0.9141)^2}{(8)(0.0784)}} = 0.0383$$

   then antilog (SE) is the multiplicative standard error of $\hat{EC50}$, i.e.,

   $\hat{EC50}\ \overset{\times}{\div}$ antilog (SE).

   antilog SE (0.0383) = multiplicative error of EC50 = 1.092

   EC50 = 6.42 $\overset{\times}{\div}$ 1.092, $\left(\frac{6.42}{1.092}\right.$, (6.42) (1.092), one standard error limit